BASIC RESEARCH

Silencing SMYD3 in hepatoma demethylates RIZI promoter induces apoptosis and inhibits cell proliferation and migration

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Abstract

AIM: To investigate the role of SMYD3 in hepatocellular carcinoma (HCC) development and progression and to verify whether its regulation activity was through RIZ1 inactivation.

METHODS: Expression of SMYD3 in HCC cell lines and tissues were measured; silencing of SMYD3 by RNA interference (RNAi) was effectuated, hepatoma cell proliferation, migration and apoptosis were tested, with RIZ1 CpG promoter methylation, and corresponding mRNA expression were investigated.

RESULTS: SMYD3 over-expression in HCC was associated with RIZ1 hypermethylation and mRNA down-expression. Suppression of SMYD3 expression de-methylated RIZ1 CpG promoter ($P < 0.01$) and increased RIZ1 mRNA expression ($P < 0.01$). Consequently, SMYD3 down-expression with RIZ1 de-methylation strongly inhibited hepatoma cell growth (MTT inhibitory rates: Pgenesil-1-s1 60.95% ± 7.97%, Pgenesil-1-s2 72.14% ± 9.68% vs Pgenesil-1-hk 6.89% ± 4.12%, $P < 0.01$) and migration (Pgenesil-1-s1 4.24% ± 1.58%, Pgenesil-1-s1 4.87% ± 0.73% vs Pgenesil-1 19.03% ± 4.63%, Pgenesil-1-hk 19.95% ± 5.21%, $P < 0.01$) and induced apoptosis (FCM subG1 phase Pgenesil-1-s1 19.07% ± 1.78%, Pgenesil-1-s2 17.68% ± 2.36% vs Pgenesil-1 0.47% ± 0.12%, Pgenesil-1-hk 1.46% ± 0.28%, $P < 0.01$. TUNEL-positive cells: Pgenesil-1-s1 40.24% ± 5.18%, Pgenesil-1-s2 38.48% ± 4.65% vs Pgenesil-1 2.18% ± 1.34%, Pgenesil-1-hk 2.84% ± 1.22%, $P < 0.01$) in HepG2 cells.

CONCLUSION: These results demonstrate that SMYD3

plays a critical role in the carcinogenesis and progression of HCC. The proliferation, migration induction and apoptosis inhibition activities of SMYD3 may be mediated through RIZ1 CpG promoter hypermethylation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, generally long-term survival is disappointing. Epigenetics, defined as heritable changes in gene expression that are not coded in the DNA sequence itself, is increasingly linked with tumorigenesis[1,2]. It is well established that DNA methylation, nucleosomal histone post-translational modifications such as methylation, acetylation/deacetylation, phosphorylation ADP-ribosylation, and ubiquitination, and other epigenetic patterns are central to proper gene expression. Increasing evidence shows that disrupting epigenetic patterns can induce carcinogenesis or affect the outcome of cancer[3,4]. Among these, histone methylation is considered to be critical for transcriptional regulation and seems to play an important role in tumor epigenetic modification. Recently, SMYD3 was identified and characterized to specifically methylate histone H3 at lysine 4 (K4), and this activity is indeed dependent on an intact SET domain, an evolutionarily conserved protein module shown to facilitate histone methyltransferase activity[5]. Expression of SMYD3 was frequently enhanced in colorectal carcinoma (CRC), breast cancer tissue, as well as HCC, and elevated *SMYD3* expression was involved in the growth of CRC, breast cancer and HCC cells[5-7]. Although transcriptional activation of downstream genes including *Nkx2.8* and *WNT10B* gene has been reported[5,6], the effect of SMYD3 on HCC development and the underlining mechanism remains unclear.

Inactivation of tumor suppressive genes (TSGs) plays an important role in carcinogenesis. RIZ1, a typical

TSG with H3-K9 methyltransferase activity, was shown to lose its expression and tumor-suppressing activity in many types of human tumors including HCC[8], CRC[9], breast cancer[10], prostate cancer[11] and gastric cancer[12]. Adenovirus-mediated RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis in breast cancer, liver cancer, and microsatellite instability-positive colon cancer cells. Adenovirus RIZ1 can also inhibit growth of colon cancer xenografts[13]. Previous data suggest that the RIZ locus is a target of frequent deletion in HCC, but a more common way of RIZ inactivation in HCC may not involve mutations that alter peptide sequences, but by CpG promoter hypermethylation[14].

Therefore, we sought to explore whether targeting of SMYD3 expression can inhibit the development of HCC, and determine whether the biological function of SMYD3 in HCC development and progression is mediated through RIZ1 inactivation. We found SMYD3 was significantly overexpressed in HCC cell lines, but not in normal hepatocellular cells and peri-cancerous tissues. Silencing of SMYD3 by RNAi remarkably inhibited HCC cell proliferation and migration, and induced apoptosis *in vitro*. Furthermore, RIZ1 was under-expressed in HCC cells with its promoter hypermethylated, while inhibition of SMYD3 demethylated RIZ1 and recovered its expression in HCC cells. Our results indicated that SMYD3 plays pro-oncological function in HCC development and progression; thus the activity of SMYD3 in HCC may be partly through its activity on RIZ1 promoter hypermethylation and RIZ1 inactivity.

MATERIALS AND METHODS

Cell lines
HCC cell lines HepG2 and Hep3B were purchased from the American Type Culture Collection (Rockville, MD). HCC cell line SMMC-7721 and liver cell line L-02 were generous gifts from Dr. Danhui Weng (HUST, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco-BRL).

RT-PCR
Total RNA was extracted using Trizol reagent (Takara, Tokyo, Japan). RT-PCR was performed with the Advantage RT-PCR kit (Takara), according to the manufacturer's protocol. PCR setting: Initial denaturation at 94℃ for 3 min before 18 cycles (for *GAPDH*) or 30 cycles (for *SMYD3* and *RIZ1*) at 94℃ for 30 s, 50℃ for 45 s and 72℃ for 60 s. The sets of primers are listed in Supplementary Information Table S1.

shRNA construction and transfection
The Pgenesil-1 vector containing the U6 promoter region was purchased from Genesil Biotechnology Corporation (Wuhan, China). Plasmids expressing siRNAs were prepared by cloning double-stranded oligonucleotides into the Pgenesil-1 vector. The sets of primers are listed in Supplementary Information Table S1. Positive clones were identified by restriction digestion and confirmed by

sequencing. The resulting plasmids were designated as Pgenesil-1-s1, Pgenesil-1-s2 and negative control Pgenesil-1-hk. Transfection was performed by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol.

Western blot analysis
Proteins were prepared by homogenization of cells in lysis buffer (10 mmol/L Tris-HCl, pH 8.0; 140 mmol/L NaCl; 5 mmol/L EDTA; 0.25 g/L NaN$_3$; 10 g/L Triton X-100; 10 g/L deoxycholate; 1 g/L SDS; 0.5 mmol/L PMSF; 1 g/L leupeptin; 1 g/L aprotinin). Protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA). Proteins were resolved (30 μg per lane) in 120 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transblotted onto a PVDF membrane (Amersham, Arlington Heights, IL). Membranes were blocked in 50 mL/L non-fat milk overnight and subsequently incubated with 1:2000 rabbit polyclonal anti-SMYD3 antibody (a generous gift from Dr. Ryuji Hamamoto of the University of Tokyo, Tokyo, Japan), or anti-β-actin (1:5000 dilution, Sigma, St. Louis, MO) for 1 h and then with secondary anti-rabbit IgG-horseradish-peroxidase antibody for 1 h at room temperature. Immunoreactive proteins were visualized by means of enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) and exposure to autoradiographic film.

Proliferation assay of HepG2 cell
MTT experiments were carried out using the cell proliferation kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's manual. Briefly, stable transfected and parental HepG2 cells were seeded at a density of 5×10^3 cells/well in 96-well plates and allowed to grow for 48 h and then 20 μL 5 g/L MTT was added. After incubation for 4 h, DMSO 200 g/L was added to each well to dissolve crystals. After 5 min incubation at 37℃, absorbance was measured at 570 nm. Assays were performed in triplicate.

Migration assay of HepG2 cell
This assay for the invasiveness of cells was based on the principle of Boyden chamber (BD Biosciences, San Diego, CA) and performed according to the manufacturer's protocol. Briefly, the top compartment was prepared by coating the filter with diluted Matrigel and incubated for 30 min. A suspension of 1×10^4 cells in serum-free medium was inoculated in the upper chamber, and conditioned media obtained from NIH3T3 cells was placed in the lower compartment of the chamber, as a chemoattractant. After 24 h incubation, noninvasive cells were removed with a cotton swab. The cells which migrated through the filter and adhered to the lower surface of the filter were fixed with methanol, stained with hematoxylin, and counted manually in 5 randomly selected microscopic fields. Assays were performed in triplicate. The data were reported as the percentage of cells successfully passing through the Matrigel and filter relative to those migrating through the control filter.
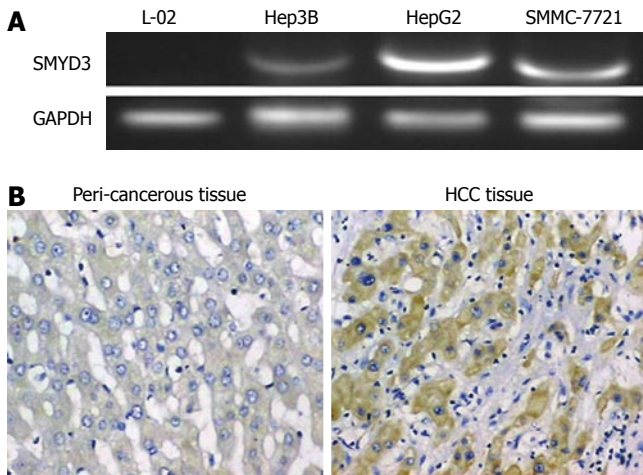
Figure 1 Enhanced expression of SMYD3 in HCC. **A**: The expression of SMYD3 genes was determined by RT-PCR and GAPDH served as an internal control; **B**: Representative images of immunohistochemical staining of accumulated SMYD3 protein in HCC tissue, but not in peri-cancerous tissue.

Figure 2 RNAi specifically inhibits SMYD3 expression in HCC cell lines. **A**: Schematic drawing of the Pgenesil-1 vector; **B**: The predicted secondary structures of the Pgenesil-1-s1 and Pgenesil-1-s2 transcripts target SMYD3 are shown; **C**: 48h after transfection, Western blot analysis showed the inhibitory effect of plasmids expressing SMYD3 shRNAs in HepG2 and SMMC-7721 cells.

FCM analysis

HepG2 cells, 1×10^6, were seeded in 60-mm dishes and transfected with SMYD3-RNAi-plasmids at 60%-80% confluence. Then, 48 h after transfection, cells were deprived of serum for 36 h. Afterwards, cells were harvested, fixed in 700 mL/L ethanol and washed with $1 \times$ PBS and suspended in 50 mg/L propidium iodide. DNA content was determined by flow cytometry using a Becton-Dickinson FACSCalibur (Becton Dickinson, Bedford, MA). Results were analyzed by ModFit LT2.0 and Cellquest software. Assays were performed in triplicate.

TUNEL assay

Apoptotic cells were identified with the *In Situ* Cell Death Detection kit (Roche Applied Science, Shanghai, China) using the protocol recommended by the manufacturer. In brief, HepG2 cells were grown on coverslips. The next day, cells were transfected with SMYD3-RNAi-plasmids. At 48 h after transfection, cells were deprived of serum for 36 h. Coverslips with adherent cells were fixed in 40 g/L paraformaldehyde for 1 h at room temperature and permeabilized with 1 g/L Triton X-100 for 2 min on ice. DNA fragments were labeled with the TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture for 60 min at 37℃ in a humidified atmosphere in the dark. Diaminobenzidine was used to mark the apoptotic cells (brown staining). Cells were counted manually in six randomly selected microscopic fields for each sample. The apoptosis index was defined by the percentage of apoptotic cells among the total cells of each sample.

RIZ1 promoter methylation specific PCR assay

Methylation Specific PCR (MSP) assay was performed according to the procedure described by Fang *et al*[15]. In brief, 1 μg of the genomic DNA was modified by sodium bisulfide using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) in accordance with the manufacturer's instructions. Modified DNA was amplified by two d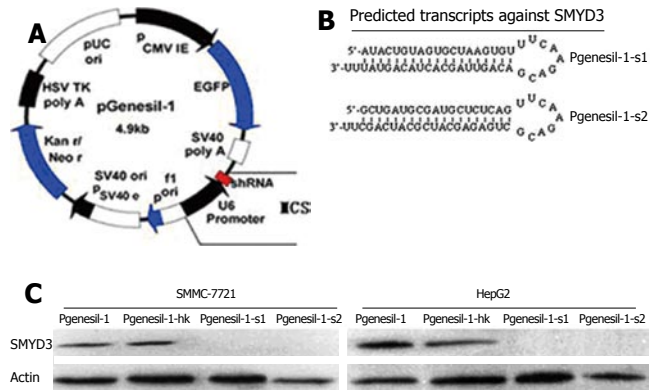ifferent primer pairs specific to the unmethylated (U) and methylated (M) *RIZ1* sequences, respectively. The sets of primers are listed in Supplementary Information Table S1. The PCR amplification was performed for a total of 45 cycles with an annealing temperature of 68℃ and 60℃ for M-sequences and U-sequences, respectively. The PCR products were then analyzed using a 35 g/L agarose gel.

Statistical analysis

SPSS 13.0 was used to analyze the data. Statistical significance was assessed by comparing mean ± SD with Student's *t* test for independent groups. $P < 0.05$ was considered statistically significant.

RESULTS

Enhanced SMYD3 expression in HCC cell lines

To determine whether SMYD3 gene is overexpressed in HCC cell lines, we compared the level of SMYD3 gene expression in normal human hepatocellular cell line L-02 to that of three HCC cell lines. RT-PCR analysis revealed that SMYD3 was overexpressed in all HCC cells, but absent in L-02 cells (Figure 1). These data indicate that enhanced SMYD3 expression is involved in a majority of HCC.

RNAi specifically inhibits SMYD3 expression in HCC cell lines

To inhibit SMYD3 expression in HCC cells, we used a DNA-based siRNA method (Figure 2A). Two siRNAs targeting different 21 sequences of human SMYD3 were cloned into Pgensil-1 vector to express RNA, which is expected to fold back to form a hairpin loop structure after being transcribed. The hairpin dsRNA can then be further cleaved by Dicer to generate a 21-nucleotide siRNA, the active form for the RNAi effect (Figure 2B). We used HepG2 and SMMC-7721 cells, which overexpress SMYD3 abundantly. Forty eight hours after transfection, Pgenesil-1-s1 and Pgenesil-1-s2 markedly knocked down SMYD3 expression in these cells, as determined by Western blot analysis. The specificity of RNAi targeting SMYD3 was shown by transfection with Pgenesil-1-hk which had no effect on SMYD3 expression (Figure 2C).
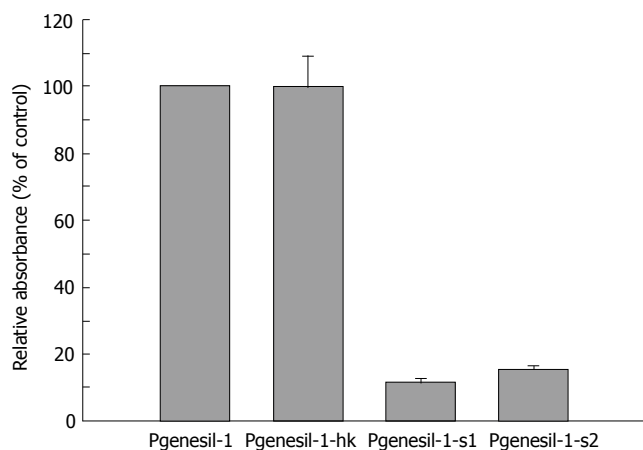
Figure 3 Inhibition of SMYD3 reduces hepatoma cell proliferation (MTT assays).

Figure 4 Inhibition of SMYD3 reduces hepatoma cell migration. **A**: Representative cells (blue) invade through Matrigel and membrane; **B**: The percentage of cells successfully passing through the matrigel and membrane (*P* < 0.01).

Inhibition of SMYD3 reduces hepatoma cell proliferation

MTT assay was used to examine the inhibitory effect of RNAi against SMYD3 on cell growth in HepG2 cells. Suppression of SMYD3 expression significantly inhibited cell growth compared to the cells transfected with control plasmid Pgenesil-1. Pgenesil-1-hk, which did not suppress expression of SMYD3, was shown to have little inhibitory effect on growth of HepG2 cells, compared to Pgenesil-1 (Figure 3). The growth inhibitory effect of the plasmids was consistent with their gene silencing effect. Therefore, SMYD3 RNAi significantly suppressed the growth of HeG2 cells *in vitro*, indicating that SMYD3 may be involved in the regulation of cell proliferation.

Inhibition of SMYD3 reduces hepatoma cell migration

The HepG2 cell line has been characterized as a highly invasive hepatocellular cancer cell line. To determine whether SMYD3 gene knockdown by RNAi could reduce its invasive potential, an *in vitro* invasion assay was performed. As shown in Figure 4, HepG2 cells were greatly deprived of invasiveness by depletion of SMYD3 gene expression. The percentage of invasive cells was decreased by 3-4 fold in Pgenesil-1-s1 and Pgenesil-1-s2 treated groups, compared to Pgenesil-1 treated groups. No significant decrease of invasive cells was observed in Pgenesil-1-hk treated cells. These results demonstrate that SMYD3 not only plays a pivotal role in the process of development, but it also is involved in tumor cell migration.

Inhibition of SMYD3 promotes apoptosis in hepatoma cells

Flow cytometry analysis showed that after 48 h of transfection and 36 h serum deprivation, the number of cells in the sub G1 phase was 19.07 ± 1.78% and 17.68 ± 2.36% in HepG2 cells transfected with Pgenesil-1-s1 and Pgenesil-1-s2, respectively, while only 0.47% ± 0.12% and 1.46% ± 0.28% cells were observed in HepG2 cells transfected with Pgenesil-1 and Pgenesil-1-hk, respectively (Figure 5A). These results possibly suggest the induction of apoptosis. To confirm whether silencing of SMYD3 can induce apoptosis in HCC cells following serum deprivation, TUNEL 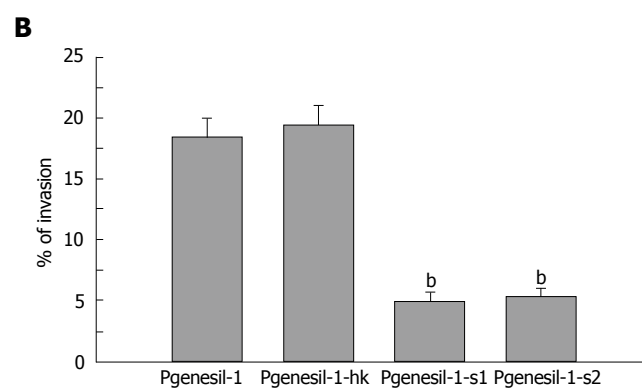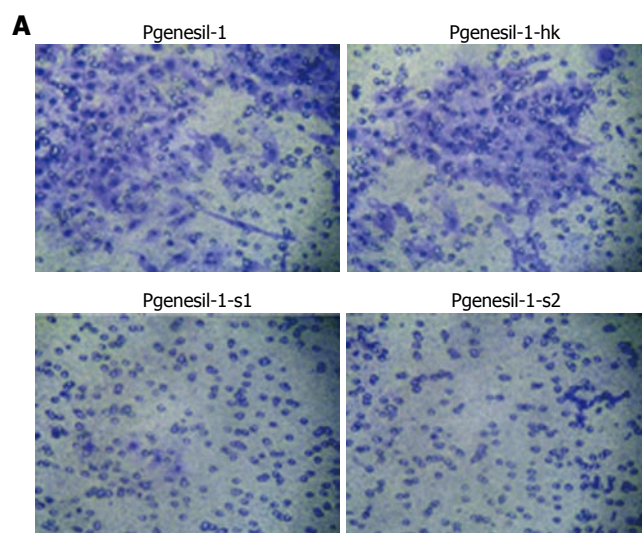assay was performed. The number of TUNEL-positive cells was 40.24% ± 5.18% and 38.48% ± 4.65% in HepG2 cells transfected with Pgenesil-1-s1 and Pgenesil-1-s2, respectively. Only 2.18% ± 1.34% and 2.84% ± 1.22% were TUNEL positive in control cells transfected with Pgenesil-1 and Pgenesil-1-hk, *P* < 0.01, respectively (Figure 5B). These results indicate that inhibition of SMYD3 by RNAi significantly promoted apoptosis in HepG2 cells following serum deprivation.

Inhibition of SMYD3 de-methylates RIZ1 promoter CpG islands and re-expresses RIZ1 in hepatoma cells

Through MSP and RT-PCR, we observed that the RIZ1 promoter was totally methylated with the lack expression of RIZ1 mRNA in HepG2 cells. After inhibition of SMYD3 by Pgenesil-1-s1, the RIZ1 promoter was found partial methylated in HepG2 cells, consistent with the change of RIZ1 mRNA (Figure 6). These results suggest that the activity of SMYD3 may be regulated through RIZ1 CpG promoter hypermethylation and RIZ1 inactivation.

DISCUSSION

Among epigenetic regulatory ways, histone methylation, perhaps more than any other form of modification, has demonstrated the power of modifications over DNA-
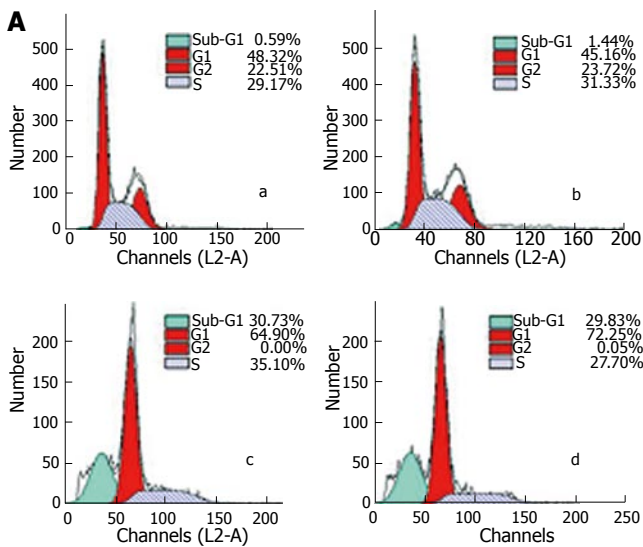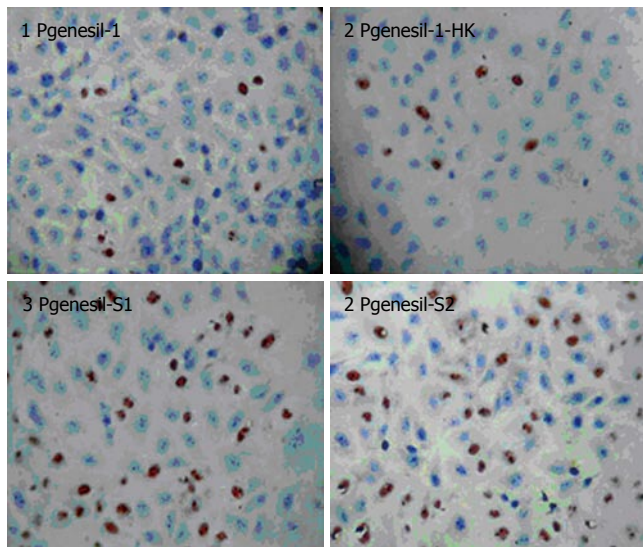
Figure 5 Inhibition of SMYD3 promotes apoptosis in hepatoma cells upon serum deprivation. **A**: FCM analysis of HepG2 cells 48 h after transfection (*P* < 0.01). **B**: TUNEL assay of cell apoptosis with siRNA suppression of SMYD3 expression in HepG2 (brown staining cells) (*P* < 0.01).

based functions, regulating fundamental processes such as gene transcription and DNA repair[16]. SMYD3, a gene located in 1q44 chromosome, encodes a protein with HMTase activity specifically on histone H3 at K4, and is involved in carcinogenesis and tumor progression[5]. In the present study, we confirmed that SMYD3 is overexpressed in HCC cell lines. To further investigate the function of SMYD3 in HCC carcinogenesis and tumor progression, RNAi technology was applied to specifically knock down SMYD3 expression in HCC cell lines. We demonstrated that depletion of SMYD3 reduced hepatoma cell growth, migration and induced cell apoptosis upon serum deprivation. Our findings suggest that SMYD3 plays pro-oncologic role in HCC development and progression. Interestingly, our results show that the expression level of SMYD3 in Hep3B cells differs significantly from that in HepG2 and SMMC-7721 cells. We hypothesized that the difference is possibly due to the HBV infectious state
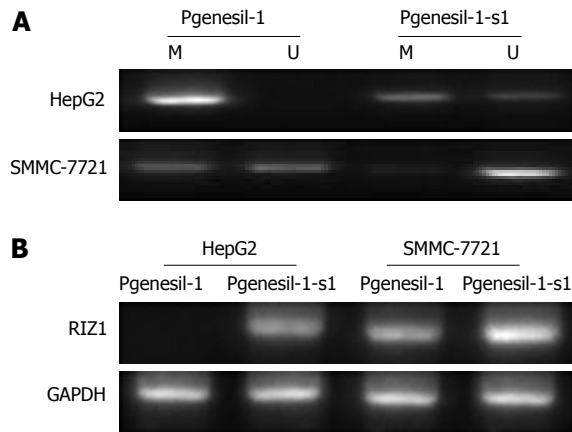

Figure 6 Inhibition of SMYD3 de-methylates RIZ1 promoter CpG islands and reexpresses RIZ1 in hepatoma Cells. **A**: Methylation specific PCR (MSP) assay of RIZ1 promoter. M: Methylated; U: Unmethylated; **B**: RIZ1 mRNA expression, GAPDH served as internal control (RT-PCR).

of the cells that we used; HBV positive in Hep3B, but negative in HepG2 and SMMC-7721 cells. However, this hypothesis needs to be further clarified.

SMYD3 is considered to function through its H3-K4 histone metylation, regulating expression of Nkx2.8[17,18], Wnt10B[19,20] and other genes involved in hepatoma cell-cycle regulation, cell proliferation and apoptosis. SMYD3 also upregulates genes linked to cell adhesion and invasion, such as ITGA5[21], COLQ[22], SELL[23], NEURL[24], and PECAM1[25], which may be responsible for cell migration regulatory role of SMYD3 in our experiment. To investigate whether SMYD3 might act on HCC oncological activity through TSG inactivation, we examined if RIZ1 activity could be regulated by SMYD3 in different hepatoma cells. RIZ1 was found to be downregulated in HepG2 cells with its promoter CpG hypermethylated, which was in line with enhanced SMYD3 expression, while knockdown of SMYD3 demethylated RIZ1 promoter and upregulated RIZ1 expression in these cells. In addition, different SMYD3 expression levels in hepatoma cells was also consistent with RIZ1 promoter methylation and mRNA expression in hepatoma cells in our results (data not shown) and in other results. HepG2 cells have been shown to lack RIZ1 expression due to promoter hypermethylation; in contrast, Hep3B cells do not show *RIZ1* promoter hypermethylation and express RIZ1 mRNA[26,27]. These results strongly suggest that SMYD3 plays a role in HCC development and progression, partly through RIZ1 promoter hypermethylation and RIZ1 inactivation.

As a histone/protein methyltransferase, SMYD3 mainly acts on histones or proteins; therefore, the mechanism by which SMYD3 regulate RIZ1 promoter CpG islands needs to be further clarified. SMYD3 includes a putative 428-amino acid protein containing a SET domain (codons 148-239) which is HMT, and a zf-MYND domain (codons 49-87), a typical zinc finger domain. The presence of a MYND-type zinc-finger domain in SMYD3 suggests that SMYD3 can recognize and bind particular sequences present in the promoter region of downstream genes through its MYND zinc finger. The specific SMYD3

binding elements (SBE) in target DNA are 5'-CCCTCC-3' or 5'-GGAGGG-3', which are present in the promoter regions of SMYD3 downstream genes, such as Nkx2.8[5]. It is interesting to note that one SBE, 5'-GGAGGG-3', is present in the promoter region of RIZ1[27]. A typical zinc finger domain in SMYD3 and SBE sequence in RIZ1 promoter strongly suggests that SMYD3 may act on RIZ1 promoter through its zf-MYND domain recognizing and binding to the SBE within the RIZ1 promoter. However, SMYD3 may also recognize SBE in other genes, such as DNA methyltransferases (DNMT), which are crucial for DNA methylation[28], and sequentially regulates RIZ1 promoter hypermethylation. There is other evidence suggesting that histone modification may interact with DNA methylation or may regulate DNA methylation[29]. In mammals, H3K9 methylation and CpG methylation shows a complex interplay in which each mark can influence the activity of the other.

Our results imply that, besides their gene-transacting role, SMYD3 and other H3-K4 methyltransferases might influence carcinogenesis and tumor progression by silencing TSGs through DNA methylation. In mammals, DNA methylation must be catalyzed by DNA methyltransferases, whether SMYD3 directly regulates DNMT expression, or SMYD3 changes the local conformation of RIZ1 promoter to facilitate DNMT congregation needs to be further investigated. Moreover, SMYD3 can di- and tri-methylate H3-K4, if its DNA methylation activity is dependent on H3-K4 transactivation, the H3-K4 hypermethylation patterns also needs to be established.

COMMENTS

Background
SMYD3, a H3K4 methyltransferase, was shown to be enhanced expressed in HCC, and involved in the growth of hepatocellular carcinoma (HCC) cells. Although transcriptional activation of downstream genes including Nkx2.8 and WNT10B gene was reported, the effect of SMYD3 on HCC development and the underlining mechanism remain unclear. RIZ1, one typical TSG with H3-K9 methyltransferase activity, was shown to lose its expression and tumor-suppressing activity in HCC, RIZ inactivation in HCC was mainly through its CpG promoter hypermethylation. In this article, whether SMYD3 regulates HCC proliferation, apoptosis and migration through RIZ1 promoter hypermethylation was initially investigated.

Research frontiers
In previous studies, histone metylation were thought to be irreversible. However, in recent studies, histone demethylase was found, which means that histone can be hypermethylated or demethylated in regulating gene expression. Since histone modification play important epigenetic regulating roles in gene transcription, this may provide new target for carcinoma therapy through hypermethylating or demethylating histone.

Innovations and breakthroughs
This article suggests H3-T4 histone methyltransferase regulates HCC biology, not only through oncogene transcription, but through interacting with H3-K9 HMT which mainly plays inhibitory role in oncogene expression, and through inhibiting TSG expression by promoter hypermethylation. These findings deepen our understanding of the interaction of different histone modification ways in gene transcription modification.

Applications
Clarifying the regulating models of H3-K4 methyltransferase, such as SMYD3, represents a potentiality for understanding oncogene expression and tumor suppressive gene under-expression in carcinogenesis and progress. By deepened

understanding the key role of SMYD3 in HCC development may also provide new target for carcinoma treatment.

Terminology
SMYD3 SET and MYND domain-containing protein 3; RIZ1 retinoblastoma protein-interacting zinc finger gene1; TSG tumor suppressive gene; HMT Histone methyltransferase; DNMT DNA methyltransferases.

Peer review
The manuscript describes studies showing that several HCC cell lines and one HCC tissue sample express SMYD3 and is well described.

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